Genetic encoding of non-natural amino acids in *Drosophila melanogaster* Schneider 2 cells

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Abstract: Insect cells are useful for the high-yield production of recombinant proteins including chemokines and membrane proteins. In this study, we developed an insect cell-based system for incorporating non-natural amino acids into proteins at specific sites. Three types of promoter systems were constructed, and their efficiencies were compared for the expression of the prokaryotic amber suppressor tRNA^{Tyr} in *Drosophila melanogaster* Schneider 2 cells. When paired with a variant of *Escherichia coli* tyrosyl-tRNA synthetase specific for 3-iodo-L-tyrosine, the suppressor tRNA transcribed from the U6 promoter most efficiently incorporated the amino acid into proteins in the cells. The transient and stable introductions of these prokaryotic molecules into the insect cells were then compared in terms of the yield of proteins containing non-natural amino acids, and the “transient” method generated a sevenfold higher yield. By this method, 4-azido-L-phenylalanine was incorporated into human interleukin-8 at a specific site. The yield of the azido-containing IL-8 was 1 µg/1 mL cell culture, and the recombinant protein was successfully labeled with a fluorescent probe by the Staudinger–Bertozzi reaction.

Keywords: aminoacyl-tRNA synthetase; amber suppressor tRNA; interleukin-8; azido group; site-specific protein modification

Introduction

The repertoire of genetically encoded amino acids has been expanded in *Escherichia coli*,¹ mammalian cells,² and yeast,³ for the purpose of increasing the chemical and structural diversity of proteins. These developments have enabled the cell-based production of proteins with “non-natural” amino acids at specific sites, called “alloproteins”.⁴ These amino acids have

novel structures not found in the canonical amino acids, such as a heavy atom,^{2,5} a fluorescent probe,⁶ photo-induced crosslinkers,^{7,8} chemical “handles” for site-specific protein modification,^{9–11} bulky groups tethered with a long linker,^{11,12} a hydroxyl group in place of the α-amino group,^{13,14} and naturally occurring post-translational modifications.^{15,16} The incorporation of these unique functionalities facilitates protein science and engineering.

Non-natural amino acids have not been genetically encoded in insect cells, although these cells are widely used for the large-scale preparation of recombinant proteins. Insect cells are useful for producing such proteins that cannot be expressed in *E. coli* and yeast cells, and are difficult to produce in large amounts in mammalian cells. A variety of cell lines have been developed from different insect sources. The Sf9 cell line from *Spodoptera frugiperda*

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Figure 1. Chemical structures of L-tyrosine and its derivatives.

and the HighFive cell line from *Trichoplusia ni* have been successfully used to prepare mammalian proteins, including G protein-coupled receptors,¹⁷ whereas *Drosophila melanogaster* Schneider 2 cells (S2 cells) have been used for the preparation of secreted proteins with sugar chains, including human cytokines¹⁸ and antibodies.¹⁹

The genetic encoding of a non-natural amino acid relies on the pair of an amber suppressor tRNA and a variant of the aminoacyl-tRNA synthetase (aaRS) specific to the amino acid. This aaRS-tRNA pair must not crossreact with any endogenous tRNA or aaRS species; otherwise, the non-natural amino acid would be incorporated at other sites in addition to the amber codon, while the amber codon would also be translated into canonical amino acids. This required “orthogonality” of the aaRS-tRNA pair to the host translation system has been achieved by using exogenous aaRS-tRNA pairs.^{1,2,20} For example, 3-iodo-L-tyrosine (Fig. 1) has been genetically encoded in mammalian cells with the pair of a specific variant of tyrosyl-tRNA synthetase (TyrRS) from *E. coli* and the amber suppressor tRNA^{Tyr} from *Bacillus stearothermophilus*.²

Such aaRS variants, like other recombinant proteins, can be expressed using conventional expression vectors, while the expression of prokaryotic tRNA species in mammalian or yeast cells requires the development of appropriate systems.^{2,12,21,22} Although the suppressor tRNAs from *D. melanogaster*^{23,24} and *Schizosaccharomyces pombe*²⁵ reportedly caused nonsense suppression in insect cells, prokaryotic tRNAs have not been expressed in these cells. To develop an expression system, the following two factors must be considered. One is the internal promoter within a tRNA gene, which works to transcribe the gene in eukaryotic cells and is absent from most of the prokaryotic tRNA genes. The *B. stearothermophilus* amber suppressor tRNA^{Tyr} has been successfully utilized in mammalian cells,^{2,26,27} because this tRNA happens to have the internal promoter sequence.

Another factor is the sequence at the 5' side of the tRNA coding sequence. Three types of such upstream sequences have been used for prokaryotic tRNA expression. One is the upstream sequence of a

human tRNA^{Tyr} gene, which was connected to the *B. stearothermophilus* suppressor tRNA^{Tyr} gene to enhance its expression in mammalian cells.² The second type is the H1 and U6 promoters, which were originally located upstream of RNA coding sequences, and have been placed before the genes encoding *E. coli* tRNA^{Tyr}, tRNA^{Leu}, and archaeal tRNA^{Pyl}, to achieve their efficient expression in eukaryotic cells.^{12,21} Note that these tRNA species all lack the internal promoter. The third type includes a eukaryotic tRNA sequence with the internal promoter¹² and the internal promoter sequences isolated from the small nucleolar RNA and RNase-P RNA genes. These sequences have also been placed before the prokaryotic tRNA genes lacking the internal promoter, to facilitate their expression in eukaryotic cells.²²

In this study, we tested different systems for expressing a prokaryotic amber suppressor tRNA in *D. melanogaster* S2 cells. The *E. coli* suppressor tRNA^{Tyr} transcribed from the U6 promoter generated the highest yield of alloproteins, although the *B. stearothermophilus* tRNA^{Tyr} was also useful in the insect cells. The *E. coli* TyrRS-suppressor tRNA^{Tyr} pair was shown to be orthogonal to the insect aaRS-tRNA system, and 3-iodo-L-tyrosine and 4-azido-L-phenylalanine (Fig. 1) were successfully incorporated into the proteins produced by cells transiently transfected with the plasmids. The feasibility of stably maintaining the genetic system for encoding non-natural amino acids was also demonstrated with S2 cells.

Results

Development of an expression system for prokaryotic amber suppressor tRNA in S2 cells

We developed an expression system for a prokaryotic suppressor tRNA, by monitoring the site-specific incorporation of 3-iodo-L-tyrosine into proteins, using the tRNA and a TyrRS variant. This non-natural amino acid has been incorporated in mammalian cells expressing the *B. stearothermophilus* suppressor tRNA^{Tyr} and a 3-iodo-L-tyrosine-specific variant of *E. coli* TyrRS,²⁸ designated as iodoTyrRS-ec. We tested this pair in *D. melanogaster* S2 cells with the following two modifications. First, instead of using iodoTyrRS-ec, we used another variant, designated as iodoTyrRS-ed,²⁹ which is derived from the former enzyme and shows greatly improved specificity for 3-iodo-L-tyrosine. Second, the upstream sequence connected to the prokaryotic tRNA gene was changed from that of the human tRNA^{Tyr} gene to that of the *D. melanogaster* tRNA^{Tyr} gene²³ [Fig. 2(A)]. The *lacZ* gene with an amber mutation at position 91, *lacZ*(Am91), was used to detect the amber suppression, with 3-iodo-L-tyrosine (1 mM) supplementation of the growth media. The genes encoding the

Figure 2. Development of the expression system for prokaryotic suppressor tRNA^{Tyr} in S2 cells. A: The three expression systems for the bacterial suppressor tRNA^{Tyr} molecules. “Dm” means *D. melanogaster*. “TTTTT” indicates the transcription terminator. B: The relative amber suppression efficiencies of the three expression systems, measured in terms of the β-gal activity in the transiently transfected S2 cells. The bar above “vector” shows the β-gal activity with neither LacZ(Am91), iodoTyrRS-ed, nor suppressor tRNA^{Tyr}. The bar above “-tRNA” shows the β-gal activity without suppressor tRNA^{Tyr}. The white bar shows 5% of the β-gal activity due to the wild-type LacZ transiently expressed in the cells. C: A map of the plasmid pAcEYR/iodoTyrRS-ed. D: The amber suppression due to the U6 promoter system for expressing the *E. coli* suppressor tRNA^{Tyr}. “-tRNA,” “-aaRS,” and “-IY” mean the absence of the suppressor tRNA^{Tyr}, iodoTyrRS-ed, and 3-iodo-L-tyrosine, respectively. Each bar represents the average of three independent experiments throughout this figure.

suppressor tRNA, iodoTyrRS-ed, and LacZ(Am91), each carried by a different vector, were transiently introduced into S2 cells. The amber suppression occurred at an efficiency of 1% relative to the yield of the wild-type LacZ in S2 cells [Fig. 2(B)]. The removal of 3-iodo-L-tyrosine from the growth media abolished the amber suppression (data not shown), indicating that this non-natural amino acid was incorporated at the amber position.

This finding showed that the *B. stearothermophilus* tRNA^{Tyr} gene is useful for genetically encoding non-natural amino acids in not only mammalian cells but also insect cells. We then tested another strategy involving external promoters, to explore the possibility of improving the suppression efficiency. We constructed two more gene constructs, each including the *E. coli* amber suppressor tRNA^{Tyr} gene instead of the *B. stearothermophilus* tRNA^{Tyr} gene, because the internal promoter in the bacillus tRNA gene may interfere with the transcription from external promoters [Fig. 2(A)]. The first construct contained the *D. melanogaster* U6-2 promoter, which was previously used for the expression of short hairpin RNA in S2 cells,³⁰ placed at the 5' end of the *E. coli* suppressor tRNA^{Tyr} sequence. This construct, designated as U6-EYR, efficiently caused amber suppression, and the yield of the suppression product of lacZ(Am91) was 6% of that of the wild-type LacZ, in

terms of the β-galactosidase activity [Fig. 2(B)]. The second construct consisted of a tRNA^{Tyr} gene from *D. melanogaster*, together with its upstream sequence, and the *E. coli* tRNA^{Tyr} sequence. These two regions were separated by a short linker sequence. The fly tRNA gene was found to allow very little expression of the downstream *E. coli* tRNA^{Tyr} gene [Fig. 2(B)].

Since U6-EYR worked best among the tested constructs, to optimize the suppression efficiency, three copies of U6-EYR were carried in tandem on a vector and used to produce alloproteins. We previously reported that the alloproteins yield was improved by increasing the copy number of the suppressor tRNA gene.^{2,12} The suppression efficiency due to three copies of U6-EYR was found to be 1.8-fold greater than that due to one copy, in terms of the β-galactosidase activity.

Finally, we examined the orthogonality of the iodoTyrRS-ed and *E. coli* suppressor tRNA^{Tyr} pair in S2 cells. Three copies of U6-EYR and the iodoTyrRS-ed gene were cloned in the same plasmid, pAcEYR/iodoTyrRS-ed [Fig. 2(C)], in which the enzyme variant was expressed constitutively from the actin 5C promoter. S2 cells were then transfected with this plasmid, together with another plasmid carrying lacZ(Am91). Amber suppression with an efficiency of 12% was observed in the presence of 3-iodo-L-

Figure 3. Enrichment of the stably transfected S2 cells. (A) Schematic illustration of the applied procedure. For details, refer to Materials and Methods section. (B–E) S2 cells harboring pLY-LacZ(Am91) (B, D) and pLY-LacZ (C, E) were stained with X-gal at the selection steps indicated in panel A. The percentage of stained cells is shown above each panel.

tyrosine [Fig. 2(D)]. When either the suppressor tRNA or iodoTyrRS-*ed* was removed from the plasmid, the amber suppression was hardly detectable, showing that both the prokaryotic tRNA and TyrRS were necessary for the suppression. In the absence of 3-iodo-L-tyrosine, the suppression efficiency was reduced from 12% to 0.4%, showing that iodoTyrRS-*ed* is specific for the non-natural amino acid, whose occupancy at the amber codon was estimated to be more than 95%.

Development of S2 cells stably maintaining the genetic system encoding 3-iodo-L-tyrosine

S2 cells have been stably transfected with recombinant genes to achieve the high-yield production of target proteins, because hundreds of copies of the expression cassette can be introduced into chromosomes, and the strong metallothionein promoter is available for the inducible expression of recombinant proteins.³¹ Therefore, we attempted to stably maintain the iodoTyrRS-*ed* and *E. coli* suppressor tRNA^{Tyr} pair in S2 cells for alloprotein production. Since it is difficult to isolate a single S2 cell and grow it further, cells stably maintaining a certain plasmid can only be enriched in a polyclonal cell population. First, we created plasmids pLY-LacZWT and pLY-LacZ(Am91) by cloning the wild-type *lacZ* gene and the *lacZ*(Am91) gene, respectively, in a plasmid carrying the iodoTyrRS-*ed* gene and three copies of U6-EYR. All of the protein genes were placed under the control of the metallothionein promoter. To select cells

stably maintaining these plasmids, S2 cells were also transfected with the plasmids harboring marker genes, in addition to the above plasmids. One of the markers was the constitutively expressed puromycin resistance (*pac*) gene, while the other was an amber mutant of the blasticidin S-resistance (*bsr*) gene, placed under the control of the methallothionein promoter. The selection was performed in two steps, using these antibiotics [Fig. 3(A)].

S2 cells were incubated for 2 weeks in the presence of puromycin. After this first selection, 38% of the cells transfected with pLY-LacZ exhibited the β -galactosidase activity, as judged by X-gal staining, while 7% of the cells with pLY-LacZ(Am91) displayed the activity [Fig. 3(B,C)]. Provided that the fraction of cells actually transfected with the plasmid is similar between these two plasmids, the difference in the percentage of stained cells should mean that some of the cells harboring pLY-LacZ(Am91) could not support a detectable level of LacZ production, probably because the low-expression level of iodoTyrRS-*ed* or the suppressor tRNA. To enrich the cells with high-level amber suppression, we performed the second selection, which was dependent on the suppression of the amber mutant *bsr* gene.

The first trial of the second selection was performed by inducing the expression of iodoTyrRS-*ed* and the mutant *bsr* gene, with 3-iodo-L-tyrosine and blasticidin supplemented in the growth media. However, the cells exhibiting the β -galactosidase activity were not enriched, probably because even poor

Figure 4. Production of IL-8 variants containing 3-iodo-L-tyrosine and 4-azido-L-phenylalanine in S2 cells. (A, B) SDS-polyacrylamide electrophoresis of the purified IL-8 and its variants with the non-natural amino acids. Five- and 10-percent amounts of the obtained wild-type IL-8 were applied for comparison. The product of the amber mutant IL-8 gene was obtained in the presence of 3-iodo-L-tyrosine (+IY) and 4-azido-L-phenylalanine (+AzF), while it was scarcely obtained in the absence of these amino acids (-IY, -AzF). (C) A fluorescent image of the SDS-polyacrylamide gel of panel B, obtained with an LAS-1000 image analyzer (Fujifilm, Japan).

expression of the *iodoTyrRS-ed-tRNA^{Tyr}* pair supported a sufficient expression of the mutant *bsr* gene for cell survival. Therefore, we decided to take advantage of the marginal level of suppression caused by the *iodoTyrRS-ed-tRNA* pair in the absence of non-natural amino acids. As described earlier, this pair introduced into S2 cells transiently caused the suppression at 0.4% efficiency without 3-iodo-L-tyrosine. Only the cells efficiently expressing the *iodoTyrRS-ed-tRNA* pair were expected to support the required expression level of the mutant *bsr* gene in the absence of 3-iodo-L-tyrosine.

After the second selection was performed by this modified procedure, 39% of the cells with *pIY-LacZ*(Am91), which survived for about 50 days after transfection, exhibited β -galactosidase activity in the presence of 3-iodo-L-tyrosine [Fig. 3(D)]. On the other hand, the cells with *pIY-LacZ* were also subjected to the second selection, and the population with the β -galactosidase activity significantly increased, from 38 to 60% [Fig. 3(E)]. This observation suggested that not every cell that had survived the puromycin selection harbored the *pIY-LacZ* plasmid, and that the population of the cells with *pIY-LacZ* was increased after the second selection, which required the activity of the *iodoTyrRS-ed-tRNA* pair.

Finally, we compared the alloprotein yields between the stably and transiently transformed S2 cells. The cell population enriched with the cells stably maintaining *pIY-LacZ*(Am91) was incubated for 3 days in the presence of 3-iodo-L-tyrosine, while the transiently transfected population was also incubated for 2 days with the non-natural amino acid. Cell extracts were prepared from 2×10^6 cells of each population, for the determination of the β -galactosidase activity. We found that the yield of the amber suppression product from the "stable cell" population was 14% of that from the "transient cell"

population. By staining the stable cell population with X-gal, we found that the proportion of stably transformed cells dropped from 39% to 14%, during the subculturing process before alloprotein production. This drop in proportion occurred despite the supplementation of the growth medium with blastidicin. Subculturing is a necessary step to generate a cell population sufficient for the large-scale production of a recombinant protein, and a reduction in the proportion of stably transformed cells is often seen with S2 cells. It seems that this problem must be overcome to obtain a large amount of alloprotein from the stable cell population.

High-yield alloprotein production in S2 cells

We demonstrated the high-yield production of an alloprotein, using S2 cells transiently transfected with the plasmid *pAcEYR/iodoTyrRS-ed* [Fig. 2(C)]. We incorporated 3-iodo-L-tyrosine into human interleukin-8 (IL-8) at the amber position located just before the C-terminal tag sequence. The yield was 5 μ g of the purified alloprotein per 2.4 mL cell culture, which demonstrated a high yield of alloprotein based on the transient transfection method [Fig. 4(A)]. We then incorporated another non-natural amino acid, 4-azido-L-phenylalanine (AzF), into proteins. This amino acid is available for site-specific protein modification, and can be incorporated by its specific variant of *E. coli* TyrRS,³ designated as AzFRS. The *iodoTyrRS-ed* gene in the plasmid was replaced by the gene encoding AzFRS. The full-length IL-8 was produced when AzF was present in the growth media, whereas the product was hardly detected without the non-natural amino acid [Fig. 4(B)]. The yield of the AzF-containing IL-8 was determined as 2.2 μ g/2.4 mL cell culture, which was 4.5% of the yield of the wild-type IL-8. This alloprotein yield is essentially comparable to the yield that we

Figure 5. MALDI-TOF analysis of the IL-8 derivatives before (A) and after (B) the labeling reaction with a triarylphosphine-biotin conjugate. The peaks X, Y, and Z correspond to the IL-8 molecules with 4-aminophenylalanine and AzF, and the biotin-labeled IL-8 molecule, respectively.

previously achieved with human embryonic kidney 293 cells.¹²

Site-specific protein modification of the azido-containing IL-8 was performed by the Staudinger–Bertozzi reaction,³² using triarylphosphine conjugated with fluorescein.¹¹ After the reaction with the fluorescent probe, the native IL-8 and the azido-containing variant were analyzed using a fluorescence imager, and only the band corresponding to the variant was observed [Fig. 4(C)]. Finally, we conducted mass spectrometric analyses before and after the labeling of the azido-containing IL-8 with a biotin-triarylphosphine conjugate. The mass spectrum before the reaction shows two major peaks, X and Y, corresponding to the IL-8 molecules with one 4-aminophenylalanine residue and with one AzF residue, respectively [Fig. 5(A)]. The amino group is probably derived from the azido group by degradation after translation. The two minor peaks, y1 and y2, corresponded to the phosphorylated and sugar-attached IL-8 variants with AzF, judging from their average mass. Thus, the mass spectrometric data revealed that the produced full-length IL-8 contained only AzF at the amber position. After the labeling reac-

tion, the biotin-labeled IL-8 variant was detected as the major peak, Z, with its phosphorylated and sugar-attached derivatives in the minor peaks z1 and z2, respectively [Fig. 5(B)]. On the other hand, the AzF-containing IL-8 variant was found as a minor peak, showing that the majority of the azido-containing IL-8 was labeled in the Staudinger–Bertozzi reaction. The IL-8 with 4-aminophenylalanine was still detected, because it cannot react with the reagent.

Discussion

In this study, we developed a *D. melanogaster* S2 cell-based system for producing proteins with non-natural amino acids at specific sites. This is the first report describing the genetic encoding of such amino acids in insect cells, although an insect cell-free translation system has been used to incorporate non-natural amino acids into proteins site-specifically.³³ Three different gene constructs were designed, based on the factors known to be critical for the expression of a prokaryotic suppressor tRNA in eukaryotic cells. Although the *B. stearotherophilus* tRNA^{Tyr} gene, with the upstream sequence of the fly tRNA^{Tyr} gene, was able to function in S2 cells, the *E. coli* suppressor tRNA^{Tyr} transcribed from the fly U6 promoter exhibited more efficient amber suppression. The introduction of multiple copies of the U6-tRNA gene increased the alloprotein yield, as also found in mammalian cells. On the other hand, the tandem gene construct consisting of the fly tRNA^{Tyr} gene followed by the *E. coli* suppressor tRNA^{Tyr} gene hardly worked in the insect cells, although a similar construct successfully expressed the prokaryotic tRNA^{Tyr} to produce alloproteins in yeast cells.²² Yeasts naturally have tandem tRNA genes, and a yeast tRNA gene has been used to transcribe exogenous genes in yeast cells,³⁴ whereas no tandem tRNA genes have been found in the fly.

The stable maintenance of a system to genetically encode non-natural amino acids in mammalian cells has not been achieved. Although an aaRS variant gene was stably maintained in Chinese hamster ovary cells, the paired suppressor tRNA was not stably expressed in the cells.² This study demonstrated the feasibility of the stable maintenance of an aaRS–suppressor tRNA pair for a non-natural amino acid in S2 cells. Since the S2 cell line does not allow single-cell isolation, it is only feasible to enrich stably transfected cells in the polyclonal cell population. This drawback has two undesirable effects: it is difficult to increase the proportion of stably transfected cells to nearly 100%, and this proportion often drops during subculturing. Our selection procedure, which takes advantage of the amber suppression due to the introduced iodoTyrRS-*ed* and suppressor tRNA^{Tyr} pair, enriched the stably transfected cells to 40% of the total. Although the transiently transfected cell

population yielded a significantly larger amount of alloprotein, future improvements of the enriching and subculturing procedures will also make stably transfected cells useful for the high-yield production of alloproteins.

S2 cells have been used to produce chemokines, cytokines, and antibodies for both protein analyses and therapeutic purposes. The site-specific protein modification by the incorporation of the azido group can be utilized to add polyethylene glycol to proteins, to increase their stability for use as therapeutic agents.³⁵ Antibodies may be labeled with a fluorescent probe at specific sites that do not interfere with antigen binding. We recently showed that the site-specific incorporation of 3-iodo-L-tyrosine facilitates protein X-ray crystallography,³⁶ and this method can now be applied to proteins prepared with S2 cells. The incorporation of other useful non-natural amino acids besides tyrosine derivatives, as well as the use of other insect cell lines, such as Sf9 and HighFive, will be the targets of further study. Our achievement with S2 cells will facilitate the further development of alloprotein-producing systems with insect cells.

Materials and Methods

Plasmids

Three prokaryotic tRNA^{Tyr} genes were each constructed on the pCR4 Blunt-TOPO vector (Invitrogen). First, the *D. melanogaster* U6-2 promoter sequence was amplified by PCR from pDU62P³⁰ and then linked to the 5' end of the *E. coli* suppressor tRNA^{Tyr} sequence, without the 3'-CCA trinucleotide,² to create U6-EYR. Second, the sequence of the *D. melanogaster* tRNA^{Tyr} gene from positions -123 to +73, with position +1 corresponding to the 5' end of the tRNA,²³ was obtained from S2 chromosomal DNA extracted using a yeast genome extraction kit, Dr. GentLE (Takara, Japan), and was then placed upstream of the *E. coli* suppressor tRNA^{Tyr} sequence with a 24-base linker sequence, AGATCTCA-TAAAAAACAATAAAT. Third, the 123-nucleotide upstream sequence of the *D. melanogaster* tRNA^{Tyr} starting from position -1 was attached to the 5' end of the *B. stearothermophilus* suppressor tRNA^{Tyr}. The *lacZ* and amber mutant *lacZ*(Am91)³⁷ genes were substituted for the *lacZ* gene in the pMT/V5-His/*lacZ* vector (Invitrogen), to create the plasmids pLacZ and pLacZ(Am91), respectively. The *E. coli* TyrRS variant iodoTyrRS-*ed*²⁹ was cloned into the pMT/V5-HisA and pAc5.1/V5-HisA vectors (Invitrogen) to create pMT/iodoTyrRS-*ed* and pAc/iodoTyrRS-*ed*, respectively. The sequence encoding human IL-8 in the processed form (lacking the first 27 amino acids), capped by four amino acids (RYGG) and C-terminally tagged with a FLAG and a 6 × His sequence in this order, was cloned just after the BiP secretion signal (MKLCILLAVVAFVGLSLG) in the

pAcBiP vector,³⁸ to create pAcBiP/IL-8. An amber codon was inserted just before the FLAG tag in pAc-BiP/IL-8, to create pAcBiP/IL-8(Am).

To construct the plasmids carrying the genes encoding the TyrRS variants, the suppressor tRNA^{Tyr}, and the reporter LacZ, three copies of U6-EYR were cloned at the SalI sites of pMT and pAc5.1 to create pMTEYR and pAcEYR, respectively. The gene encoding iodoTyrRS-*ed* was cloned into pMTEYR and pAcEYR to create pMTEYR/iodoTyrRS-*ed* and pAcEYR/iodoTyrRS-*ed*, respectively, whereas the AzFRS gene was cloned into pAcEYR to create pAcEYR/AzFRS. The *lacZ* and *lacZ*(Am91) genes were cloned into the ScaI site of pMTEYR/iodoTyrRS-*ed*, to create pIY-LacZ and pIY-LacZ(Am91), respectively.

The blasticidin S-resistance gene was cloned into the pMT vector, and the 38th codon was then mutagenized to the amber codon to create pMT/*bsr*(Am). The amber suppression product of this mutant gene containing 3-iodotyrosine in *E. coli* cells retains its activity. The puromycin resistance gene with the copia promoter, obtained from pCoBlast (Invitrogen), was cloned into the pGL3 vector (Promega), to create the plasmid pCoPur. The puromycin resistance gene and the promoter, obtained from pCoPur, were cloned into the SalI site of the pMT/*bsr*(Am) to create pMT/*bsr*(Am)/*pac*.

Amber suppression in S2 cells

The *Drosophila* S2 cells (Invitrogen) were cultured in Schneider's *Drosophila* medium (Invitrogen) containing 10% fetal bovine serum. Plasmid transfection was performed using 24- or 6-well plates. The following details in the transfection procedure refer to the use of a 24-well plate; fourfold larger amounts of cells and reagents were used with a 6-well plate. First, 0.5×10^6 cells in 0.5 mL of the growth medium were seeded in each well of a 24-well plate at 27°C for 24 h before transfection using Effectene (Qiagen). The plasmids (0.5 µg in total) were gently dissolved using a Vortex mixer for 1 s in EC buffer (75 µL) supplemented with the enhancer (8 µL), both from Qiagen, and this plasmid solution was incubated at room temperature for 5 min. The solution was mixed with 20 µL of Effectene by gently vortexing for 10 s, and was then incubated at room temperature for 15 min. The Effectene-plasmid solution was mixed with 125 µL of fresh medium for addition to the cells in each well, just after the growth medium in the well was replaced with 375 µL of fresh medium. After 2-h incubation, the growth medium was supplemented with penicillin, streptomycin, and non-natural amino acids. As a supplement in the medium, 3-iodo-L-tyrosine (Sigma) was dissolved in 0.1% acetic acid to a final concentration of 20 mM. 4-Azido-L-phenylalanine (Bachem) was dissolved in 0.2N HCl and then neutralized with 10N KOH, to

prepare a solution with a final concentration of 10 mM. To induce the expression from the metallothionein promoter, a CuSO₄ solution (100 mM) was added to the growth media to a final concentration of 500 μM. The β-galactosidase assay was performed as described.³⁷ The X-gal staining of S2 cells was performed using a beta-galactosidase staining kit (Mirus Bio LLC, Madison, WI). The produced human IL-8 was purified from the recovered media by the batch method using Ni-Sepharose 6 fast flow (GE Healthcare). The Staudinger–Bertozzi reaction was performed as described previously.¹¹

Stable transfection

The S2 cell culture (5 × 10⁶ cells), in 5 mL of growth medium, was transfected with pCoPur and pMT/*bsr*(Am)/*pac* and either pIY-LacZ(Am91) or pIY-LacZWT, at a weight ratio of 1:1:19, respectively, with a total plasmid amount of 5.25 μg. After an incubation for 4 days at 27°C, the cells were collected by centrifugation to completely remove the old growth medium, which was replaced by fresh medium containing 10 μg/mL puromycin (Invitrogen). When the number of cells increased and some of them were found floating in the medium, the cell culture was diluted with fresh medium containing puromycin (10 μg/mL). After further incubation for 2 weeks with further dilutions by the puromycin-containing media, the CuSO₄ solution was added to induce the expression of the amber mutant *bsr* gene and iodoTyrRS-*ed*, but 3-iodo-L-tyrosine was not supplemented in the medium at this stage. After 3 or 4 days, blasticidin S (Invivogen) was added to the cell culture at a final concentration of 25 μg/mL. When the medium became crowded with growing cells, the culture was diluted eightfold with medium lacking blasticidin S, for a healing cycle for 3 or 4 days, followed by an induction with CuSO₄ for 3 or 4 days. The blasticidin selection and healing cycle were repeated one more time before cell staining with X-gal, or three more times before the β-gal assay.

Labeling reaction and mass spectrometric analysis

The Staudinger–Bertozzi reaction was performed according to the procedure previously described.¹¹ Triarylphosphine-fluorescein and triarylphosphine-biotin conjugates were purchased from Shinsei Chemical Company Ltd. (Osaka, Japan). Mass spectrometric analysis was commercially performed by Shimadzu Biotech (Japan). IL-8 molecules in the labeling reaction mixture were treated with a Zip Tip pipette tip (Millipore) before the analysis.

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